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Loss of Retrovirus Production in JB/RH Melanoma Cells Transfected with H-2K^b and TAP-1 Genes

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JB/RH1 melanoma cells, as well as other melanomas of C57BL/6 mice (B16 and JB/MS), express a common melanoma-associated antigen (MAA) encoded by an ecotropic melanoma-associated retrovirus (MelARV). JB/RH1 cells do not express the H-2K^b molecules due to down-regulation of the H-2K^b and TAP-1 genes. When JB/RH1 cells were transfected with the H-2K^b and cotransfected with the TAP-1 gene, it resulted in the appearance of H-2K^b molecules and an increase in their immunogenicity, albeit they lost expression of retrovirus-encoded MAA recognized by MM2-9B6 mAb. Loss of MAA was found to result from a complete and stable elimination of ecotropic MelARV production in the H-2K^b/TAP-1-transfected JB/RH1 cells. Northern blot analysis showed no differences in ecotropic retroviral messages in MelARV-producing and -nonproducing melanoma cells, suggesting that loss of MelARV production was not due to down-regulation of MelARV transcription. Southern blot analysis revealed several rearrangements in the proviral DNA of H-2K^b-positive JB/RH1 melanoma cells. Sequence analysis of the ecotropic proviral DNA from these cells showed numerous nucleotide substitutions, some of which resulted in the appearance of a novel intraviral *Pst*I restriction site and the loss of a *Hin*dIII restriction site in the *pol* region. PCR amplification of the proviral DNAs indicates that an ecotropic provirus found in the H-2K^b-positive cells is novel and does not preexist in the parental H-2K^b-negative melanoma cells. Conversely, the ecotropic provirus of the parental JB/RH1 cells was not amplifable from the H-2K^b-positive cells. Our data indicate that stable loss of retroviral production in the H-2K^b/TAP-1-transfected melanoma cells is probably due to the induction of recombination between a productive ecotropic MelARV and a defective nonecotropic provirus leading to the generation of a defective ecotropic provirus and the loss of MelARV production and expression of the retrovirus-encoded MAA.

INTRODUCTION

We have reported previously that the B16 melanoma and its sublines, as well as two other melanomas (JB/RH and JB/MS) of C57BL/6 mice, express the melanoma-associated antigen (MAA) recognized by MM2-9B6 mAb (Leong *et al.,* 1988; Gorelik *et al.,* 1995). However, melanomas of other strains of mice (Cloudman S91 of DBA/2, K1735 of C3H, and Harding-Passey of wild-type mice) as well as various normal and malignant cell lines (carcinomas, sarcomas, fibrosarcomas, and lymphomas) of C57BL/6 mice do not express this MAA, indicating that this MAA is tissue- and strain-specific (Leong *et al.,* 1988). The MM2-9B6 mAb has been used effectively in the eradication of established lung and liver metastases of B16 and JB/MS melanomas (Eisenthal *et al.,* 1987; Hearing *et al.,* 1991). Analysis of the nature of this MAA revealed that it is a product of an ecotropic C-type retrovirus (Leong *et al.,* 1988). Recently we found that MAA is encoded by the *env* gene of an ecotropic C-type retrovirus produced in melanomas of C57BL/6 mice (Li *et al.,* 1996). The genome of C57BL/6 mice contains a single copy of ecotropic provirus termed Emv-2 and numerous nonecotropic proviruses: 20 xenotropic, 24 polytropic, and 13 modified polytropic proviruses (Coffin *et al.,* 1989). All these proviruses are defective. The major defect in the ecotropic Emv-2 provirus is associated with a G to C mutation at nucleotide 3576 that leads to a substitution of alanine for proline in the central portion of the encoded reverse transcriptase (King *et al.,* 1988). Our data indicate that melanomas of C57BL/6 mice (B16, JB/RH, and JB/MS) produce numerous B-tropic ecotropic retroviral particles that have been termed melanoma-associated retrovirus (MelARV) (Li *et al.,* 1996). The productive ecotropic provirus probably emerged in melanomas of C57BL/6 mice as a result of recombination between defective ecotropic Emv-2 and nonecotropic C-type proviruses. BL6 melanoma cells contain at least three copies of MelARV proviruses inserted into the different sites of their genome (Li *et al.,* 1996). Recently we tested the potential role of the melanoma-associated retroviruses in malignant transformation by infection of the cultured melanocyte lines of C57BL/6 mice with MelARV isolated from the supernatant of cultured BL6 melanoma. Two infected melanocyte lines were transformed and formed

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highly pigmented tumors in mice (Li *et al.,* 1998). Analysis of the retrovirus insertion sites revealed that in BL6 and in novel retrovirus-induced melanomas proto-oncogene *c-maf* is a common insertion site (Li *et al.,* 1998). These data suggest that C-type melanoma-associated retroviruses might play a role in melanoma formation, probably by insertion and activation of the proto-oncogene expression.

Previously we showed that BL6 melanoma cells that do not express H-2K^b molecules produce retroviral particles and express MAA recognized by MM2-9B6 mAb. BL6 clones that express the endogenous H-2K^b molecules spontaneously or after pretreatment with *N*-methyl-*N*-nitro-nitrosoguanidine or after transfection with a plasmid containing the H-2K^b gene, do not express MAA (Gorelik *et al.,* 1991). We found that loss of MAA was associated with inhibition of MelARV production due to the changes in the proviral DNA (Li *et al.,* 1996; Muller *et al.,* 1996). In addition, the H-2K^b gene positive BL6 cells showed alterations in several biological characteristics including increased immunogenicity and sensitivity to lysis by NK cells or TNF-α (Tanaka *et al.,* 1988; Gorelik *et al.,* 1990; Kim *et al.,* 1993; Itoh *et al.,* 1994) and inhibition of melanogenesis as a result of down-regulation of tyrosinase and α-melanocyte-stimulating hormone receptor gene expression (Prezioso *et al.,* 1995), reduced substrate adherence (Gorelik *et al.,* 1993), appearance of cell surface carbohydrates reacting with GS1B$_4$, SBA, or PNA lectins (Gorelik *et al.,* 1991) and loss of metastatic ability (Gorelik *et al.,* 1993). We found that the expression of GS1B$_4$ and SBA lectin binding carbohydrates in the H-2K^b gene-transfected BL6 melanoma cells is a result of up-regulation of the α1,3-galactosyltransferase gene and a reduction of cell surface sialylation leading to the unmasking of SBA binding sites (Gorelik *et al.,* 1993, 1995).

The possibility of a stable elimination of retroviral production was not previously reported in any experimental model. Therefore, stable and complete inhibition of retroviral particle production found in the H-2K^b-positive BL6 melanoma raises the question of how general this finding is and whether similar elimination of retroviral production can be induced by the MHC class I genes in other melanomas. It is also important to investigate the possible mechanisms responsible for inhibition of retrovirus production. To test this, retrovirus production in JB/RH melanoma cells was investigated. JB/RH melanoma was originally induced in C57BL/6 mice by treatment with DMBA and croton oil (Berkelhammer *et al.,* 1982). JB/RH melanoma, similar to the BL6 melanoma, produces numerous ecotropic retroviral particles that encode the MAA recognized by MM2-9B6 mAb (Leong *et al.,* 1988). In addition, JB/RH cells showed low levels of MHC class I and α-galactosyl expression (Gorelik *et al.,* 1995). Therefore, in the present study we investigated whether restoration of MHC class I molecule expression might affect MAA expression and retrovirus production in JB/RH melanoma. In addition, the nucleotide changes in the proviral DNAs of BL6 and JB/RH melanoma cells have been compared, and the mechanisms responsible for the loss of retrovirus production in these cells have been further investigated.

RESULTS

Transfection of JB/RH1 melanoma cells with the H-2K^b and TAP-1 genes. The experiments were performed using the JB/RH1 clone isolated from the parental JB/RH melanoma by limiting dilution (Gorelik *et al.,* 1995). Flow cytometric analysis showed that the JB/RH1 clone does not express the H-2K^b or cell surface carbohydrates binding GS1B$_4$ and SBA lectins, but does express a high level of MAA recognized by MM2-9B6 mAb. Using reverse transcriptase–polymerase chain reaction (RT-PCR) analysis, we found that failure of JB/RH1 cells to express MHC class I molecules is due to down-regulation of the H-2K^b and TAP-1 genes, whereas the H-2D^b gene was expressed. Pretreatment of JB/RH1 cells with IFN-γ (100 u/ml) induced expression of MHC class I genes and its products (data not shown). Thus, lack of cell surface MHC I molecules in JB/RH1 cells is probably due to down-regulation of genes encoding the heavy chains of MHC class I molecules and/or TAP-1 molecules. TAP-1 gene products play an important role in the transfer of peptides into the endoplasmic reticulum, where they bind and stabilize the heavy and light chains of the MHC class I molecules and facilitate their expression on the cell surface (Monaco, 1992). It was found that in some malignant cells lack of cell surface-detectable MHC class I molecules was due to down-regulation of TAP gene, and transfection with the TAP gene was sufficient to induce expression of MHC class I molecules in these cells (Monaco, 1992).

Therefore, to restore the H-2K^b expression, JB/RH1 cells were transfected with a plasmid containing human TAP-1 and neo^r genes. Some cells were transfected with the H-2K^b gene and cotransfected with the neo^r gene. In parallel, JB/RH1 cells were cotransfected with the H-2K^b and TAP-1 genes. Cells resistant to G418 (1 mg/ml) were subsequently selected, cloned and their expression of MHC class I molecules, MAA, and cell surface carbohydrates was analyzed (Fig. 1). The parental JB/RH1 cells do not express H-2K^b and showed very low levels of H-2D^b expression. In total, 61 clones cotransfected with the H-2K^b and neo^r genes were analyzed, and none of them showed detectable H-2K^b molecules. Analysis of 18 clones transfected with a plasmid containing TAP-1 and neo^r also were negative for H-2K^b, probably due to lack of H-2K^b gene expression. Indeed, RT-PCR analysis of RNA from several such clones showed no H-2K^b message (data not shown). However, when JB/RH1 cells were cotransfected with the H-2K^b and TAP-1 genes, 21

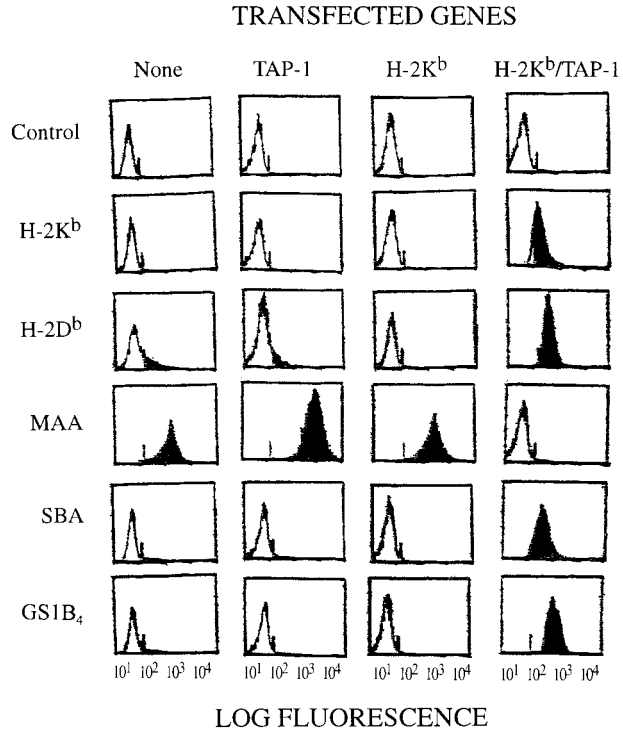

FIG. 1. The phenotype of JB/RH1 melanoma cells transfected with the H-2K^b and/or TAP-1 genes. JB/RH1 cells transfected with the H-2K^b, TAP-1, or H-2K^b and TAP-1 genes were stained with the mAbs 28-13-3 (anti-H-2K^b), 28-11-5 (anti-H-2D^b), or MM2-9B6 (anti-MAA), incubated for 30 min at 4°C, washed, and stained with the goat anti-mouse IgG conjugated with phycoerythrin. Some cells were incubated with the biotinylated GS1B$_4$ and SBA lectins and then stained with avidin conjugated with phycoerythrin. The intensity of fluorescence and percentage of fluorescent cells were analyzed by flow cytometry. The black peaks represent the specific binding of mAbs or lectins.

clones out of the 30 tested expressed high levels of H-2K^b molecules that paralleled the up-regulation of H-2D^b molecules. Expression of MHC class I was associated with some morphological changes, namely many of these cells became round and less adherent to the plastic and were capable of growing in suspension. In addition, the MHC class I-positive melanoma cells showed changes in cell surface carbohydrates with the appearance of α-galactosyl epitopes reacting with GS1B$_4$ lectin and SBA-binding carbohydrates. The representative data are depicted in Fig. 1. The parental JB/RH1 melanoma cells express high levels of MAA recognized by MM2-9B6 mAb, whereas the H-2K^b/Tap-1-transfected cells completely lost MAA expression. No changes in MAA or cell surface carbohydrate expression were found in JB/RH1 cells transfected with the H-2K^b or TAP-1 alone that did not express MHC class I molecules (Fig. 1).

It was previously shown that the MAA can be a sensitive target and that *in vivo* administration of MM2-9B6 mAb resulted in eradication of melanoma metastases that was mediated via antibody-dependent cell-mediated cytotoxicity (Eisenthal *et al.,* 1987; Hearing *et al.,* 1991). It was of interest to test whether observed loss of MAA and expression of MHC class I by JB/RH1 cells affected their immunogenicity. For this, 1×10^5 JB/RH1 cells transfected with the H-2K^b and/or TAP-1 gene were inoculated sc into C57BL/6 mice. The parental JB/RH1 cells and JB/RH1 cells transfected separately with the H-2K^b or TAP-1 genes developed progressively growing tumors with no significant differences in their rates of growth. In contrast, JB/RH1 cells that lost MAA but expressed the MHC class I molecules after cotransfection with both H-2K^b and TAP-1 genes were rejected in all five tested mice. Thus, immunogenicity of JB/RH1 cells depends primarily on expression of MHC class I-associated peptides rather than on the retrovirus-encoded and antibody-recognizable MAA.

Retrovirus production in JB/RH1 melanoma cells transfected with the H-2K^b and TAP-1 genes. MAA is encoded by MelARV, and it was of interest to test whether the observed loss of MAA in H-2K^b/TAP-1 gene-transfected JB/RH1 melanoma cells was due to down-regulation of MAA expression or to loss of retrovirus production. For this purpose, JB/RH1 melanoma cells transfected with the H-2K^b and/or the TAP-1 gene were stained with MM2-9B6 mAb and colloidal gold-labeled anti-mouse IgG antibodies and analyzed using thin-section electron microscopy. The parental JB/RH1 cells were found to produce large numbers of retrovirus particles, most of which were associated with immuno-gold-labeled MM2-9B6 mAb (Table 1). Paralleling their loss of MAA expression, all of the clones tested that had been transfected with H-2K^b and TAP-1 genes showed a loss of C-type retroviral particles that reacted with the colloidal gold-labeled MM2-9B6 mAb (Table 1). In contrast, transfection of JB/RH1 cells separately with the H-2K^b gene or TAP-1 gene alone did not lead to elimination of C-type retroviral particles or MAA expression. Similarly, no changes in retrovirus production or MAA expression were found in JB/RH1 cells transfected with neo^r or α1,3-galactosyltransferase genes (Table 1).

To further assess retroviral production in MHC class I-transfected cells, analysis of RT activity in the cell-free supernatants and in the cell lysates was performed. JB/RH1 melanoma cells showed high intracellular and extracellular RT activity that was not affected by transfection with the neo^r gene (clones RHneoC7 and RHneoC12) (Fig. 2). When JB/RH1 cells were transfected with a plasmid containing the α-1,3-galactosyltransferase and the neo^r gene (clone RHGTC42), no changes in RT activity were observed. Some reproducible reduction in RT activity inside the cells and in the supernatants was observed in clones transfected with the H-2K^b alone (clone RHKbC7) or with the TAP-1 alone (clone RHTAPC48). However, when JB/RH1 cells were cotransfected with both the H-2K^b and the TAP-1 genes, there was a complete loss of RT activity in the cells as well as in the supernatants (clones RHKTC4 and RHKTC5) (Fig.

TABLE 1

Retrovirus Production in JB/RH1 Melanoma Cells Transfected with the H-2K^b and/or TAP-1 Genes

Clones[a]	Transfected gene(s)	Flow cytometry[b] MAA Expression	Electron microscopy[c] Budding C-type particles
JB/RH1	none	+	+
RHneoC7	neo^r	+	+
RHneoC12	neo^r	+	+
RHGTC12	α1,3GT	+	+
RHGTC6	α1,3GT	+	+
RHKbC6	H-2K^b	+	+
RHKbC7	H-2K^b	+	+
RHKbC9	H-2K^b	+	+
RHTAPC48	TAP-1	+	+
RHTAPC8	TAP-1	+	+
RHTAPC5	TAP-1	+	+
RHKTC1	H-2K^b/TAP-1	−	−
RHKTC2	H-2K^b/TAP-1	−	−
RHKTC3	H-2K^b/TAP-1	−	−
RHKTC4	H-2K^b/TAP-1	−	−

[a] The melanoma clone JB/RH1 was transfected with the H-2K^b and cotransfected with the neo^r gene, or they were transfected with the H-2K^b and cotransfected with a plasmid expressing the human TAP-1 and neo^r genes. Some JB/RH1 cells were transfected only with a plasmid expressing the human TAP-1 and neo^r genes. As an additional control, JB/RH1 cells were transfected with a plasmid expressing α-1,3-galactosyltransferase cDNA and the neo^r gene. Transfection of JB/RH1 cells with the neo^r gene was also performed.

[b] Expression of MAA was analyzed using flow cytometry.

[c] Minus signs indicate ≥90% reduction in numbers of virions compared to controls (JB/RH1 melanoma cells); plus signs indicate that numbers of retroviral particles were equivalent to control.

2). Similar results were obtained when RT activities of other JB/RH1 clones transfected with the H-2K^b, TAP-1, or H-2K^b plus TAP-1 genes were tested (data not shown). Thus, analysis by electron microscopy and of RT activity indicates that loss of retrovirus-encoded MAA expression in the H-2K^b-positive JB/RH1 melanoma cells transfected with the H-2K^b and TAP-1 genes resulted from inhibition of retroviral production in these cells.

Mechanisms of inhibition of retrovirus production in JB/RH1 cells transfected with the H-2K^b and TAP-1 genes. To test whether loss of MelARV production is due to down-regulation of MelARV transcription, a Northern blot analysis was performed using RNAs extracted from the retrovirus-producing and -nonproducing H-2K^b-negative and H-2K^b-positive JB/RH1 clones. The Northern blot analysis showed that RNA isolated from JB/RH1 melanoma cells contained messages for two species of viral sequences of about 8.8 and 3.5 kb with no difference in expression of retroviral messages in any of the tested clones (Fig. 3). These data indicate that inhibition of ecotropic MelARV production in the H-2K^b/TAP-1-transfected JB/RH1 melanoma cells was not due to inhibition of retroviral transcription and that failure to produce MelARV might result from changes in the proviral DNA.

Therefore, Southern blot analysis of the proviral DNAs from JB/RH1 cells transfected with the H-2K^b and/or TAP-1 genes was performed. When high molecular weight DNA from JB/RH1 cells was digested with the *Pst*I restriction enzyme and hybridized with a ^{32}P ecotropic env-specific DNA probe, a single 8.2-kb band was found (Fig. 4). One similar fragment was generated from DNAs of JB/RH1 cells transfected with the H-2K^b gene (clone RHKbC6) or with the TAP-1 gene (clone RHTAPC5). In contrast, *Pst*I-digested DNAs from JB/RH1 cells transfected with the H-2K^b and TAP-1 genes (clones RHKTC1, RHKTC3, and RHKTC4) showed two bands: the expected 8.2-kb fragment (with reduced intensity) and a new ecotropic fragment of 5.4 kb (Fig. 4). A similar 5.4-kb fragment was also found in the H-2K^b-positive BL6-8 melanoma cells that did not produce retroviral particle (Li *et al.,* 1996; Muller *et al.,* 1996).

MelARV contains a single *Hin*dIII restriction site, and *Hin*dIII restriction enzyme cuts the ecotropic proviral DNA in the *pol* region and in the 3′ flanking cellular DNA (Fig. 5). Thus, the size of the env-containing *Hin*dIII-digested fragments depends on the provirus insertion site, and this allows the estimation of the number of ecotropic proviruses with different insertion sites. The results, shown in Fig. 6, indicate that *Hin*dIII digestion of JB/RH1 DNA generates several env-containing fragments of about 10, 7.0, and 6.0 kb with a weak band about 8.0 kb. It is of interest that BL6-8 melanoma cells also generates several *Hin*dIII-digested fragments, some of which are distinct from JB/RH1 fragments. The differences in the size and number of the ecotropic proviral *Hin*dIII-digested fragments evidence that BL6 and JB/RH are different melanomas. In clones transfected with the H-2K^b and TAP-1 genes (clones RHKTC1 and RHKTC4) the fragment smaller than 7.0 kb is missing, and a new fragment of about 9.5 kb appeared. These changes were associated with the loss of retrovirus production in these clones. Similarly, BL6-8 melanoma cells transfected with the H-2K^b gene (clone 8Kb38) showed loss of the smaller fragments (below 7.0 kb) and the appearance of two larger fragments, about 10.2 and 12.2 kb (Fig. 6, see also Li *et al.,* 1996; Muller *et al.,* 1996). JB/RH1 cells transfected separately with the H-2K^b or TAP-1 gene showed no changes in the patterns of the *Hin*dIII fragments (Fig. 6). Thus, loss of ecotropic MelARV in the H-2K^b/TAP-1-transfected JB/RH1 cells was associated with changes in the proviral sequence.

Nucleotide sequence analysis of the proviral DNA. To identify changes in the proviral DNA found in JB/RH1 cells transfected with the H-2K^b and TAP-1 genes, nucleotide sequence analysis was performed. For this purpose, ecotropic proviruses from the H-2K^b-negative JB/RH1 and H-2K^b-positive TAP-1/H-2K^b-transfected RHKTC4 cells were amplified by PCR using primers from

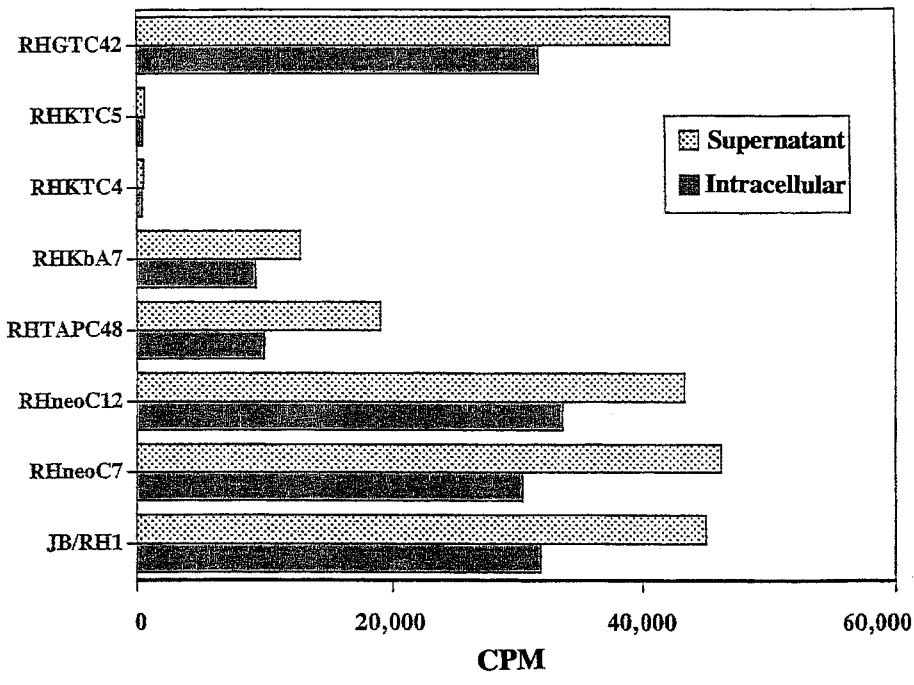

FIG. 2. Reverse transcriptase activity in cell-free supernatants and cell lysates of JB/RH1 melanoma cells transfected with H-2K^b and/or TAP-1 genes. Cell-free supernatants and cell lysates were obtained from JB/RH1 cells transfected with the neo^r gene (clones RHneoC7 and RHneoC12), with the TAP-1 gene (clone RHTAPC48), with the H-2K^b gene (clone RHKbA7), with the H-2K^b and TAP-1 genes (clone RHKTC4 and RHKTC5), or with the α-1,3-galactosyltransferase gene (clone RHGTC42). Retroviral particles contained in 1.5 ml of each supernatant were pelleted and their RT activity was assessed using the [^{3}H]TTP incorporation method. RT activity in the cell lysate per 1 $\times$ 10^6 cells is reported. Data represent one of two repetitive experiments.

the 5′ LTR and the ecotropic-specific *env* region. The resulting 6.8-kb PCR-amplified ecotropic proviral DNAs were then partially sequenced. The nucleotide sequences of the *pol* region (between 2596 and 2679 bases) of proviruses from JB/RH1 and H-2K^b-positive RHKTC4 cell variant are shown in Fig. 7. For comparison, the nucleotide sequence of the proviruses from BL6-8 and H-2K^b-positive 8Kb38 cells has been also presented. PCR products of the proviruses from the parental BL6-8 and JB/RH1 cells showed some differences, indicating that MelARVs in these cells are similar but not identical. It is of interest that the sequence CTACAG at position 2601–2606 in the *pol* gene of the JB/RH1 provirus was changed to CTGCAG in the provirus from RHKTC4 cells by a substitution of A to G. This resulted in the appearance of a novel *Pst*I restriction site in the *pol* region of the provirus in RHKTC4 melanoma cells (Fig. 7). Conversely, a *Hin*dIII restriction site, AAGCTT, of the JB/RH1-derived provirus was lost in the provirus from RHKTC4 due to a substitution of A to T and C to T at positions 2666 and 2668, respectively. It is noteworthy that the exact changes have also been found in the H-2K^b-positive 8Kb38 clone (derived from BL6-8 melanoma cells transfected with the H-2K^b gene), which explains why Southern blot analysis of proviral DNAs from the H-2K^b-positive cell variants of BL6-8 and JB/RH1 cells showed similar changes. The novel 5.4-kb *Pst*I-digested fragments of proviruses from the H-2K^b-positive BL6-8 and

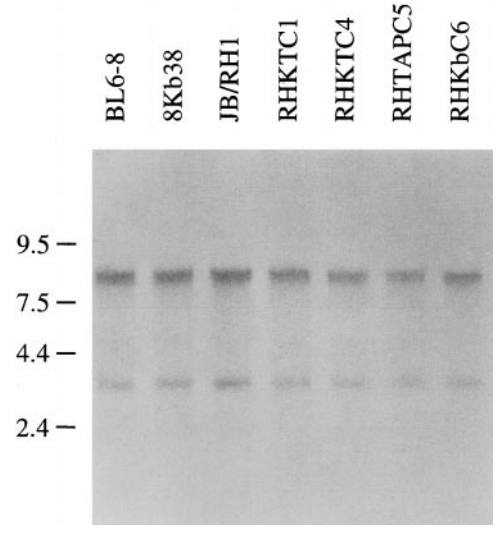

FIG. 3. Northern blot analysis of ecotropic C-type retrovirus message in JB/RH1 cells transfected with H-2K^b and/or TAP-1 genes. Total RNAs were extracted from JB/RH1 cells transfected with the H-2K^b gene (clone RHKbC6), the TAP-1 gene (clone RHTAPC5), or the H-2K^b and TAP-1 genes (clone RHKTC1 and RHKTC4). RNA was also extracted from BL6-8 melanoma cells transfected with the H-2K^b gene (clone 8Kb38). Total RNA (10 μg) extracted from melanoma cells was electrophoresed on 1% agarose–formaldehyde gel and transferred to nylon membranes by standard techniques. Transferred RNA was hybridized with a 0.4-kb ^{32}P-labeled env fragment specific for murine ecotropic retroviruses.

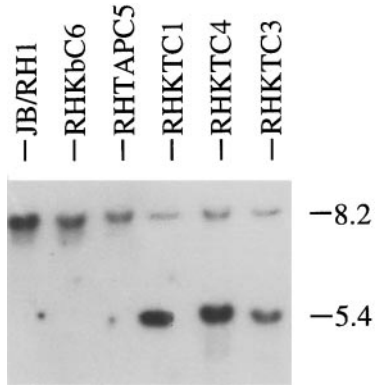

FIG. 4. Southern blot analysis of *Pst*I-digested proviral DNA isolated from JB/RH1 melanoma cells transfected with the H-2K^b and/or TAP-1 genes. DNA was extracted from various JB/RH1 melanoma subclones transfected with the H-2K^b gene (clone RHKbC6), the TAP-1 gene (clone RHTAPC5), or the H-2K^b and TAP-1 genes (clones RHKTC1, RHKTC3, and RHKTC4). DNA (10 μg) was cleaved with *Pst*I restriction enzyme and separated on a 0.6% agarose gel. DNA was transferred to nitrocellulose membranes and hybridized with an env fragment specific for ecotropic retroviruses.

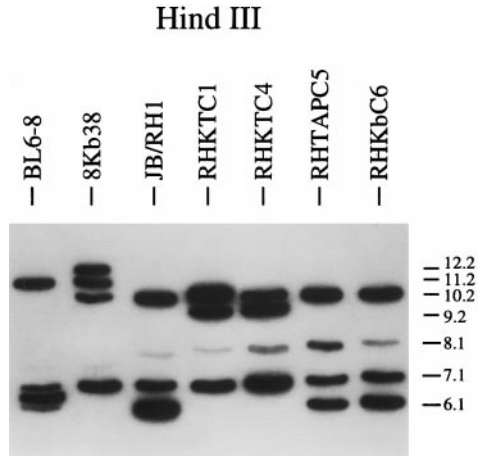

FIG. 6. Southern blot analysis of the *Hin*dIII-digested proviral DNA isolated from JB/RH1 melanoma cells transfected with H-2K^b and/or TAP-1 genes. DNA was extracted from JB/RH1 melanoma cells transfected with the H-2K^b and TAP-1 genes (clone RHKTC1 and RHKTC4), the TAP-1 gene alone (clone RHTAPC5), or the H-2K^b gene alone (clone RHKbC6). In parallel, DNAs from BL6-8 melanoma cells and BL6-8 clone transfected with the H-2K^b (clone 8Kb38) was also extracted. The DNAs were cleaved with *Hin*dIII restriction enzyme and separated on a 0.6% agarose gel. DNA was transferred to nitrocellulose membranes and hybridized with an env fragment specific for ecotropic retroviruses.

JB/RH1 cells was generated as a result of this novel intraviral *Pst*I restriction site. The *Pst*I restriction enzyme cuts the proviral DNA from these cells into two fragments (5.4 and 2.8 kb). However, only *env* containing the 5.4-kb fragment will hybridize with the ecotropic-specific env probe, whereas the 2.8-kb fragment remains undetectable with this probe. With the loss of the intraviral *Hin*dIII restriction site, the *Hin*dIII restriction enzyme cuts in the 5′ and 3′ flanking cellular sequences, resulting in the disappearance of the small fragments and the appearance of the novel larger fragments containing the ecotropic proviral DNA.

Thus, our data indicate that the loss of MelARV production in the H-2K^b/TAP-1-transfected JB/RH1 melanoma cells is associated with the alterations in a proviral DNA. The Northern blot analysis showed no differences in MelARV transcription in the retrovirus-producing and -nonproducing JB/RH1 melanoma cells (Fig. 3). It might be that in the MelARV-nonproducing H-2K^b-positive JB/RH1 cells MelARV messages are transcribed from the altered proviral DNA. To test this, RT-PCR amplification and sequencing of an ecotropic retroviral mRNAs were performed. Sequence analysis showed that the mRNA of MelARV from RHKTC4 clone transfected with the H-2K^b/TAP-1 genes substantially differs from the MelARV message from the parental JB/RH1 cells (data not shown). Actually, the nucleotide sequences of the mRNA from the MelARV-nonproducing RHKTC4 cells are complementary to the altered proviral DNA found in these cells. Indeed, the *pol* gene message contains the same nucleotide substitution, including a novel *Pst*1 restriction site and showed loss of *Hin*dIII restriction site at the same position as it was found in the proviral DNA (Fig. 7). These results suggest that elimination of ecotropic MelARV production is due to the alterations in a proviral DNA that do not hamper its expression but prevent formation of mature retroviral particles.

It is of interest that the nucleotide changes resulted in the appearance of *Pst*1 and in the loss of *Hin*dIII restric-

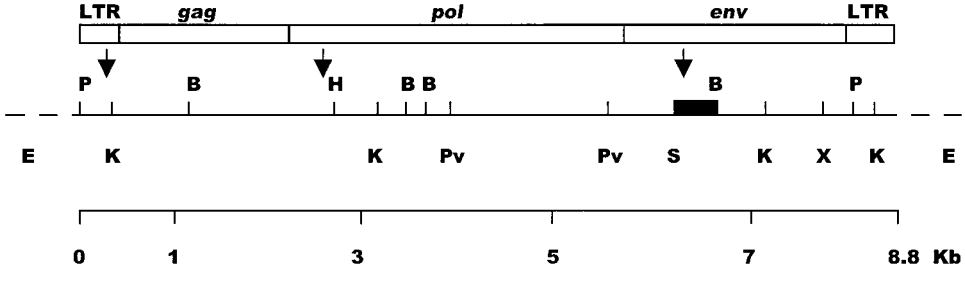

FIG. 5. Restriction sites of ecotropic MuLV proviral DNA. Abbreviations for restriction enzymes: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; X, *Xba*I (27,28). The black box indicates the position of the ecotropic-specific env fragment (Eco/env). Arrows indicate the positions of the primers used for PCR.

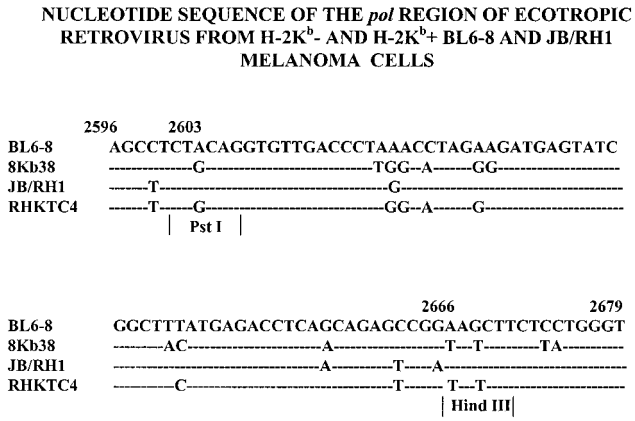

FIG. 7. Nucleotide sequences of the *pol* region of ecotropic proviruses from H-2K^b-negative and H-2K^b-positive JB/RH1 and BL6-8 melanoma cells. The position of *Pst*1 and *Hin*dIII restriction sites is indicated. H-2K^b-positive RHKTC4 clone was isolated from JB/RH1 cells cotransfected with the H-2K^b and TAP-1 genes. The H-2K^b-positive clone 8Kb38 was isolated from BL6-8 melanoma cells transfected with the H-2K^b gene.

tion sites in the MelARV proviruses from BL6-8 and JB/RH1 melanoma cells are identical (Fig. 7). The important question then is how these identical changes in two different cell lines are generated. It is unlikely that the same mutations in the identical positions at *Pst*I and *Hin*dIII restriction sites would occur in the proviruses from two independent melanoma cell lines. However, in addition to those identical changes, the proviruses from the H-2K^b-positive 8Kb38 and RHKTC4 melanoma cells showed numerous other distinct nucleotide changes. Comparison of BL6-8 and 8Kb38 proviruses between nucleotides 2596 and 2679 of the *pol* region showed 14 nucleotide differences (16.9%) (Fig. 7). The differences between the JB/RH1 and RHKTC4 proviruses were 9 out of 83 (10.8%). By contrast, comparison between the proviruses from unrelated melanoma cells BL6-8 and JB/RH1 showed fewer differences, only 5 out of 83 nucleotides (6%) (Fig. 7). We hypothesize that the differences observed in the ecotropic proviruses from the H-2K^b-negative and H-2K^b-positive melanoma clones are too numerous to be due to mutations, and most likely appeared as a result of recombination between ecotropic MelARV and xenotropic or polytropic proviruses preexisting in the genome of C57BL/6 mice.

To receive more direct confirmation of this hypothesis, PCR analysis of the ecotropic proviruses in the H-2K^b-negative and H-2K^b-positive BL6-8 and JB/RH1 melanoma cells was performed. The 3′ primer used was common to the ecotropic env-specific sequences, while the 5′ primers were selected from the *pol* region between nucleotides 2601 and 2621. The results of this analysis are presented in Fig. 8. When the 5′ primer was based on the BL6-8 provirus sequence (termed P1), a 3.7-kb PCR product was generated from BL6-8, but not from 8Kb38 proviral DNA. In contrast, when the 5′ primer used was from the pol region of 8Kb38 provirus (termed P2), a similar-sized fragment was generated only from 8Kb38, but not from BL6-8 DNA. These data indicate that the ecotropic provirus found in 8Kb38 cells is novel and it does not preexist in BL6-8 melanoma cells. Failure to amplify the BL6-8 provirus in 8Kb38 cells suggests that this provirus disappeared in these cells, probably due to recombination. Similar results were obtained with JB/RH1 melanoma cells transfected with the H-2K^b and TAP-1 genes. PCR amplification of the ecotropic provirus from the DNA of JB/RH1 cells was observed when the 5′ primer used was from the *pol* region of the JB/RH1 provirus (termed P3). However, using the same P3 primer, no PCR product was generated from the DNA of RHKTC4 cells (transfected with the H-2K^b and TAP-1 genes) that lost C-type retroviral particle production. When the 5′ primer was designed based on the pol sequences of the ecotropic provirus of RHKTC4 cells (termed P4) and was used to amplify the ecotropic proviral sequences, the 3.7-kb PCR product was generated from RHKTC4 but not from JB/RH1 DNA. Retrovirus-producing RHKbC4 cells that had been transfected with the H-2K^b gene alone showed no changes in the ecotropic provirus because the P3, but not the P4, primer was able to amplify a 3.7-kb product (Fig. 8).

These data suggest that ecotropic proviruses in RHKTC4 melanoma cells transfected with the H-2K^b and TAP-1 genes is a recombinant provirus. This provirus does not preexist in the parental JB/RH1 melanoma cells,

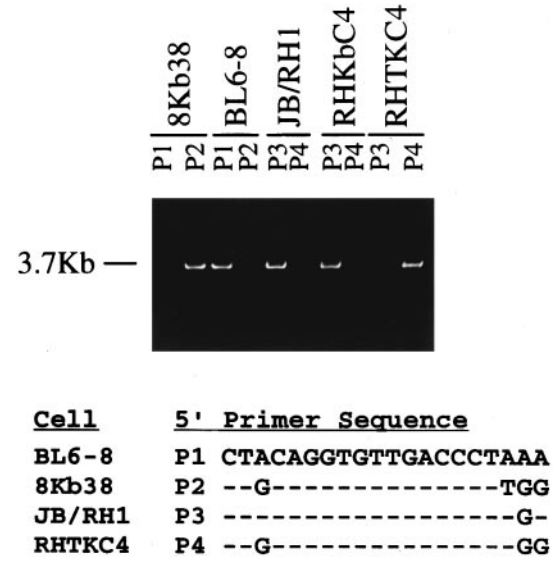

FIG. 8. PCR amplification of proviral DNAs from the H-2K^b-negative and H-2K^b-positive BL6-8 and JB/RH1 melanoma cells. Genomic DNAs isolated from melanoma cells were amplified in a long template PCR system using the different 5′ primers (P1–P4) and the common 3′ primer from the *env* of ecotropic retrovirus, as detailed under Materials and Methods. The 5′ primers P1–P4 were designed from the pol sequences (2601–2621 nucleotides) of the proviruses from JB/RH1 and BL6-8 melanoma cells, as indicated. PCR products were analyzed by electrophoresis in 0.7% agarose gel.

since no PCR product was generated from the DNA of these cells using the P4 primer. The novel recombinant ecotropic provirus found in the H-2K^b-positive melanoma cells is probably defective. Although this provirus is transcribed, it is unable to produce infectious C-type retroviral particles.

DISCUSSION

Our data show that the expression of H-2K^b molecules in JB/RH1 cells was restored only after cotransfection with both the H-2K^b and the TAP-1 genes, whereas separate transfection with the H-2K^b alone or the TAP-1 alone failed to induce the H-2K^b molecules in these cells. Appearance of the H-2K^b molecules rendered the JB/RH1 cells highly immunogenic and was associated with other phenotypic changes consistent with those previously identified in H-2K^b-positive BL6-8 melanoma cells (Gorelik *et al.,* 1991). Namely, H-2K^b-positive JB/RH1 cells showed changes in cell surface carbohydrate expression, such as the appearance of GS1B$_4$ and SBA-binding carbohydrates, and manifested a loss of MAA detectable by MM2-9B6 mAb. The loss of retrovirus-encoded MAA in the H-2K^b-positive JB/RH1 cells was resulting from loss of retrovirus production in these cells. Loss of a similar retrovirus was previously found in BL6 melanoma cells (Muller *et al.,* 1996; Li *et al.,* 1996).

Analysis of the mechanisms of inhibition of retroviral particle production in the H-2K^b-positive JB/RH1 and BL6-8 melanoma cells revealed that it was associated with changes in the proviral DNA. Nucleotide sequence analysis helped to identify the basis responsible for the observed changes. The appearance of a novel 5.4-kb fragment, following *Pst*I digestion of proviral DNA from the H-2K^b-transfected melanoma cells, is due to the emerging of a new intraviral *Pst*I restriction site in the *pol* region, whereas changes in the patterns of the *Hin*dIII-digested fragments results from a loss of a *Hin*dIII restriction site. These changes in the *Pst*I and *Hin*dIII restriction sites were identical in the BL6-8 cells transfected with the H-2K^b gene and in JB/RH1 cells transfected with the H-2K^b and TAP-1 genes. This could explain the similarity in the changes of the patterns of *Pst*I- and *Hin*dIII-digested DNA fragments identified by Southern blot analysis in these cells. However, this raises a question: how could identical nucleotide substitutions in the *Pst*I and *Hin*dIII restriction sites arise in the proviral DNA from two unrelated melanomas? An additional question concerns how these and other nucleotide changes have been introduced. First of all, changes in the nucleotide sequences in the *pol* region of MelARV from H-2K^b-positive cells are too numerous, and thus it is unlikely that they result from mutations. We hypothesize that changes observed in the proviral DNAs from H-2K^b-positive BL6-8 and JB/RH1 melanoma cells are a result of recombination between the ecotropic MelARV and endogenous nonecotropic retroviruses existing in the genome of these cells. This suggestion is supported by the following findings: (a) our PCR analysis of the proviral *pol* region indicates that the provirus found in the H-2K^b-positive melanoma cells does not preexist in the H-2K^b-negative parental melanoma cells. Conversely, the proviruses found in the parental BL6-8 and JB/RH1 cells have been changed, and thus we were unable to amplify this provirus from the H-2K^b-positive BL6-8 and JB/RH1 cells; (b) the presence of an intraviral *Pst*I and lack of intraviral *Hin*dIII restriction sites are hallmarks of the xenotropic and polytropic but not ecotropic retroviruses (Coffin *et al.,* 1989; Rands *et al.,* 1981); (c) the greatest divergence between the proviruses in the virus-producing and the virus nonproducing melanoma cells was clustered in area nt2596–nt2679 in the *pol* region (16.9%). Most interestingly, when the proviral sequence of the *pol* region of MelARV from the retrovirus-nonproducing H-2K^b-positive JB/RH1 cells was compared with that of a xenotropic retrovirus isolated from myeloma cell line SP2/0, a very high homology (about 99.8%) was found (GenBank Accession No. 94150). It is of note that xenotropic retroviruses, represented by the SP2/0-derived one, carry a *Pst*I and lack a *Hin*dIII restriction site at the same position as it was found in the modified ecotropic provirus from the H-2K^b-positive BL6 and JB/RH1 melanoma cells. It is then very likely that a similar xenotropic provirus in the melanoma genome might be one of the origins of the provirus that we cloned from the K^b+ melanoma cells. These findings strongly support the possibility that recombination between an ecotropic MelARV and a similar xenotropic provirus might create a novel ecotropic provirus that lacks the intraviral *Hin*dIII site but contains the intraviral *Pst*I restriction site in the *pol* region. This might explain the identity of changes found in the ecotropic proviral DNAs from the H-2K^b-positive BL6-8 and JB/RH1 melanoma cells. The differences in other nucleotides in the *pol* region of these proviruses might reflect the differences in the nonecotropic partners involved in this recombination in BL6-8 and JB/RH1 cells. Furthermore, such recombination between the productive MelARV and defective xenotropic retrovirus would introduce new sequences, making the new provirus defective and unable to produce mature retroviral particles. This might be a logical explanation of the observed loss of retrovirus production in the H-2K^b/Tap-1-transfected melanoma cells. Identification of the precise recombination events and the partners of this recombination is a subject of current investigation.

It is believed that productive ecotropic retroviruses from cells of C57BL/6 mice are recombinant viruses with env regions derived from the endogenous ecotropic provirus and *gag*/*pol* regions from xenotropic sequences (Kozak, 1985). Although it has not been reported previously, our data suggest that recombination may occur between productive and defective proviruses, resulting

in the generation of a novel provirus unable to produce retroviral particles. If so, directed recombination between the replication competent and defective proviruses might represent a novel approach that might lead to elimination of retroviral production.

Although the described loss of MAA and retrovirus production was observed in BL6-8 and JB/RH1 melanoma cells transfected with the H-2K^b or H-2K^b/TAP-1 genes, it is unlikely that these genes or their products could directly affect retrovirus production and expression of various cellular genes. We believe that this effect is more likely to be indirect and is probably mediated via triggering of a cascade of events, leading to enhancement of recombination between productive ecotropic and defective nonecotropic proviruses. The precise mechanisms controlling these recombinations remain unknown and need further investigations. The ability of different viruses to affect the expression of MHC class I genes has been demonstrated by numerous laboratories and has been found to be mediated by different mechanisms (Mausdley and Pound, 1991). Our studies indicate that, at least in murine melanomas, retrovirus–MHC gene interactions might also take place in the opposite direction; i.e., that expression of H-2K^b might affect retrovirus production. This experimental model might, therefore, serve as a paradigm for the analysis of mechanisms controlling retrovirus production.

MATERIALS AND METHODS

Tumor cells. The JB/RH1 clone was isolated from JB/RH melanoma by limiting dilution. The BL6-8 clone was isolated from B16BL6 (termed BL6) melanoma (Gorelik *et al.,* 1991). Clone 8Kb38 was obtained following transfection of BL6-8 cells with the H-2K^b gene (Kim *et al.,* 1993). Tumor cells were tested and found to be free of the following murine viruses: reovirus type 3, pneumonia virus, K virus, Theiler's encephalitis virus, Sendai virus, minute virus, mouse adenovirus, hepatitis virus, lymphocytic choriomeningitis, ectromelia, and lactate dehydrogenase virus (Microbiological Associates, Walkersville, MD). Tumor cells were maintained *in vitro* in RPMI 1640 medium supplemented with 10% fetal bovine serum, glutamine, streptomycin, and penicillin (all from GIBCO BRL, Gaithersburg, MD).

Mice. Female C57BL/6 mice were obtained from The Jackson Laboratory and were kept in specific pathogen-free conditions in the University of Pittsburgh Cancer Institute animal facility which is accredited for animal care by the American Association for Accreditation of Laboratory Animal Care. Mice about 2 months old were used in the experiments.

Analysis of MHC class I transcription. Total RNA was extracted as described (Li *et al.,* 1996). Four micrograms of RNA was subjected to reverse transcriptase reaction (GIBCO BRL, Grand Island, NY) in a total volume of 20 μl, using an oligo(dT) primer. Subsequently, the synthetic cDNA was used as PCR template with the addition of the specific primers and *Taq* polymerase (GIBCO BRL). PCR was performed using a sequence of 94°C, 1 min, for denaturation, 65°C, 1 min, for annealing, and 72°C, 1 min, for extension, with up to 30 cycles in total. RT-PCR was also performed with primers specific for the β-actin gene under the same conditions. The primers specific for H-2K^b, H-2D^b, TAP-1, and α_2-microglobulin genes were used (Pearce *et al.,* 1993, Levitsky *et al.,* 1994).

Gene transfection. The H-2K^b genomic fragment subcloned into the *Sal*I site of the pTCF plasmid was used for cell transfection (Tanaka *et al.,* 1988). The H-2K^b gene was transfected into JB/RH1 cells along with the neo^r gene as described (Gorelik *et al.,* 1991). In parallel, JB/RH1 cells were transfected with a plasmid containing human TAP-1 cDNA and neo^r gene that was provided by Dr. Markus Maeurer (Department of Surgery, University of Pittsburgh). Some cells were transfected with the H-2K^b gene and cotransfected with a plasmid containing TAP-1 and neo^r genes using Lipofectin reagent as previously described (Gorelik *et al.,* 1991). Cells that survived in the presence of 1 mg/ml of G418 were expanded and cloned by limiting dilution.

Monoclonal antibodies and flow cytometry. The expression of Class I H-2 antigens by tumor cells was analyzed by flow cytometry, using the following mAbs: anti-H-2K^b (hybridoma line 28-13-3 and AF6-88.53) and anti-H-2D^b (hybridoma 28-11-5). MAA was determined by MM2-9B6 (IgG$_{2b}$) mAb (Gorelik *et al.,* 1991). The MM2-9B6 mAb was developed from spleen cells of C57BL/6 mice immunized with syngeneic B16F10 melanoma cells (Leong *et al.,* 1988). Immunofluorescence staining was performed as described (Gorelik *et al.,* 1991). Flow cytometry was performed with a FACStar or FACStar-Plus flow cytometer. Cells were excited with 200 mW of the 488-nm line from an argon ion laser. Gating of the appropriate cell types was accomplished using the forward and side light scatter signals. Fluorescence signals were collected on a log scale.

Electron microscopy. Two aliquots (5×10^6 cells each) of each tested clone were prepared. One was incubated for 30 min at 4°C with 250 μl of MM2-9B6 antibody while the control sample was incubated with an irrelevant MOPC 141 antibody of the same isotype (IgG$_{2b}$). Following washing, both samples were treated for 30 min at 4°C with a 15-nm colloidal gold-conjugated rabbit anti-mouse IgG secondary antibody. After thorough washing, the cells were fixed with 2.0% glutaraldehyde–2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 3 h and stored in PBS at 4°C. Cells were postfixed with 2% osmium tetroxide, dehydrated with graded alcohol, and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate. Retroviral virions and their association with the colloidal gold particles were examined with a Zeiss EM912 Omega elec-

tron microscope. All clones were analyzed without knowledge of the phenotype of the cells (Muller *et al.,* 1996).

RT assay. Various subclones of JB/RH1 melanoma cells transfected with the H-2K^b and/or TAP-1 gene were transferred into T-25 culture flasks (1×10^6 cells in 5 ml of complete RPMI 1640 medium per flask). Three days later the supernatants of the saturated cultures were harvested, and 1.5 ml of the supernatants were distributed into microcentrifuge tubes and were centrifuged at 2000 *g* for 10 min at 4°C. The supernatants were collected, filtrated through a 0.2-μm microfilter, and then were centrifuged 14,000 *g* for 1 h at 4°C. After the supernatants were discarded, the pellets were resuspended in 10 μl of culture medium and RT activities were assessed using the [^{3}H]TTP incorporation method as described (Sei *et al.,* 1988). Samples (10 μl) were mixed with 50 μl of the RT RXN mixture containing [^{3}H]TTP (40 Ci/mmol), 10 mM EGTA, salts, and poly(rA) · $p(dT)_{12-13}$. After 2 h of incubation at 37°C, each sample was spotted onto DE81 Whatman filter circles. Specific radioactivity of the samples was measured using a liquid scintillation counter. To test intracellular RT activity, cultured JB/RH1 melanoma cells were harvested and counted. Cells were washed three times in PBS and resuspended in 200 μl of buffer C (50 mM Tris–HCl, pH 7.5, 0.25 M KCl, and 0.25% Triton X-100). After three cycles of freeze–thawing, 10 μl of cell lysates were used for RT testing using the [^{3}H]TTP incorporation method (Sei *et al.,* 1988). RT activity of cell lysates was expressed as specific radioactivity (cpm) per 1×10^6 melanoma cells (Li *et al.,* 1996).

Northern blot analysis. Northern (RNA) analysis was performed as described (Li *et al.,* 1996). Tumor cells were harvested, washed, and resuspended in 0.5% Nonidet P40–10 mM Tris–HCl (pH 7.0)–150 mM NaCl, 2 mM $MgCl_2$, incubated on ice for 3 min, and centrifugated at 1000 *g,* 4°C, for 10 min. SDS (0.5% final) and EDTA (2.5 mM final) were then added and mixed. Following sequential extractions with phenolchloroform, the RNA was precipitated and quantitated by spectrophotometric absorption. Extracted RNA was electrophoresed on 1% agarose–formaldehyde gels and transferred to nylon membranes by standard techniques. Transferred RNA was hybridized with a 0.4-kb ^{32}P-labeled env fragment specific for murine ecotropic retroviruses (probe pEc-B4) (Chan *et al.,* 1980; Chattopadhyay *et al.,* 1980). This fragment was derived from plasmid pEcB4 by digestion with *Bam*HI and *Sma*I restriction enzymes to obtain a viral-specific fragment without vector sequences (Chan *et al.,* 1980; Chattopadhyay *et al.,* 1980).

Southern blot analysis. DNA of various JB/RH1 subclones was isolated using the phenol extraction method (Li *et al.,* 1996). DNA was digested with various restriction enzymes (GIBCO BRL), and 10 μg of DNA was loaded and separated on a 0.6% agarose gel containing 0.5 μg/ml ethidium bromide. The gel was then washed for 15 min in 0.25 M HCl, 20 min in 0.5 M NaOH/1.5 M NaCl, and 30 min in 1.5 M NaCl/0.5 M Tris–HCl, pH 8.0, and blotted overnight to a Zetabind filter. The blot was prehybridized in 50% formamide, 5× SSC, 0.1 M Tris–HCl, pH 7.5, 5× Denhardt's solution, and 400 μg/ml sheared and denatured salmon sperm DNA at 42°C for 2 h in a heat-sealed bag. The blot was then hybridized with nick-translated ^{32}P-labeled (1×10^6 cpm) env fragment specific for ecotropic retroviruses (probe pEc-B4) (Chan *et al.,* 1980; Chattopadhyay *et al.,* 1980). The blot was washed with 2× SSC/0.1% SDS and then with 0.1% SSC/0.1% SDS at 68°C, and hybridized signals were autoradiographed.

PCR amplification. The genomic DNA that was isolated from H-2K^b-negative or H-2K^b-positive JB/RH1 melanoma cells was used as template in the "long template PCR system" (Boehringer Mannheim, Indianapolis, IN). The upstream and downstream primers were designed to bind to the 5′ LTR and the ecotropic virus-specific region of the env gene, respectively. The sequences of the primers were as follows: the 5′ primer from the LTR region was 5′-ACGAATTCAAGGAAGTACAGAGAGGC-3′, and the 3′ primer from the ecotropic-specific env region was 5′-AGGAATTCGATGGTTTCCAGGATG-3′. PCRs were catalyzed with *Taq* DNA polymerase plus Pwo DNA polymerase and performed with a Perkin–Elmer thermocycler 2400 as follows: starting with 94°C for 2 min for complete denaturation, followed by 10 cycles of 92°C for 10 s, 57°C for 30 s, and 68°C for 5 min and then 25 cycles of 92°C for 10 s, 57°C for 30 s, and 68°C for 5 min plus an extra elongation of 20 s/cycle. The PCR products were examined by electrophoresis on 0.7% agarose gels.

RT-PCR amplification of MelARV mRNA. The total RNAs were extracted from the parental MelARV-producing JB/RH1 and MelARV-nonproducing RHKTC4 clone transfected with the H-2K^b and TAP-1 genes. The extracted RNAs were used as templates of RT-PCR amplification. A 5′ primer from the LTR region and a 3′ primer from the ecotropic-specific *env* region were used for RT-PCR amplification. The nucleotide sequences of the primers have been presented above (see PCR amplification method). RNA (1 μg) was mixed with a set of MelARV-specific primers and then applied to the One-Step RT-PCR system plus elongase enzyme (GIBCO). The reactions were performed with a Perkin–Elmer thermocycler 2400 with the following cycling program: 50°C for 30 min, 94°C for 2 min, followed by 40 cycles of 94°C for 15 s, 58°C for 30 s, and 68°C for 7 min.

Cloning and sequencing of PCR and RT-PCR products. The 6.8-kb PCR and RT-PCR products from H-2K^b-negative JB/RH1 and H-2K^b-positive RHKTC4 subclone were recovered from the agarose gel and digested with *Eco*RI restriction enzyme (an *Eco*RI restriction site having been designed at the 5′ end of each primer). They were then cloned into an *Eco*RI cut and dephosphorylated pUC18 plasmid, respectively. DNA sequencing was accom-

plished with a "walking" strategy, starting from both ends of the inserts. The sequencing was performed using an ABI 377A automated DNA sequencer with the dideoxynucleotide chain termination method. The specific oligonucleotides used as sequencing primers were synthesized based on the sequence information obtained from the last round of sequencing. To ensure the accuracy of PCR sequencing, each PCR was independently performed twice using DNA from JB/RH1 and RHKTC4 melanoma cells as templates. Two clones derived from two independent PCRs were separately sequenced, and both strands were sequenced in two directions for each of these clones. To analyze the homology, the obtained nucleotide sequences of PCR products from JB/RH1 and RHKTC4 proviruses were compared. In addition, comparison between the nucleotide sequences of BL6-8 and 8Kb38 melanoma-derived provirus was performed. The homology of nucleotide sequences was analyzed using the PC/GENE program (IntelliGenetics, Inc., Mountain View, CA).

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